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Hemolysis and free hemoglobin revisited: Exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins

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Abstract

Hemolysis occurs in many hematologic and non-hematologic diseases. Extracellular hemoglobin (Hb) has been recognized to trigger specific pathophysiologies that are associated with adverse clinical outcomes in patients with hemolysis, such as acute and chronic vascular disease, inflammation, thrombosis and renal impairment. Among the molecular characteristics of extracellular Hb, translocation of the molecule into the extravascular space, oxidative and nitric oxide reactions, heme release and molecular signaling effects of heme appear to be the most critical. Limited clinical experience with a plasma-derived haptoglobin product in Japan and more recent preclinical animal studies suggest that the natural Hb and heme scavenger proteins haptoglobin (Hp) and hemopexin (Hpx) have a strong potential to neutralize the adverse physiologic effects of Hb and heme. This includes conditions that are as diverse as red blood cell transfusion, sickle cell disease, sepsis and extracorporeal circulation. This perspective reviews the principal mechanisms of Hb and heme toxicity in different disease states, updates how the natural scavengers efficiently control these toxic moieties, and explores critical issues in the development of human plasma-derived Hp and Hpx as therapeutics for patients with excessive intravascular hemolysis.

Introduction

When hemoglobin (Hb) bursts from red blood cells due to hemolysis, the naked Hb, devoid of its anti-oxidant sentries that are normally available within the RBC, can wreak oxidative havoc in the vasculature and in exposed tissues¹. To neutralize Hb and its reactive ferric protoporphyrin-IX group (hemin) specialized plasma scavenger proteins sequester the toxic moieties and transit them to compartments where heme-oxygenases can break down hemin into less toxic metabolites. Other molecules and reducing substances contribute to this protective physiology. However, when these clearance and detoxifying systems are overwhelmed by intravascular hemolysis such as during sickle cell disease, blood transfusion, malaria or sepsis, Hb and hemin trigger vascular and organ dysfunction that leads to adverse clinical effects (**Figure 1**). This perspective reviews the mechanisms of Hb toxicity in different disease states, updates how haptoglobin (Hp) and hemopexin (Hpx) efficiently handle free Hb and hemin and explores why the time has come to consider these proteins as therapeutics in patients with excessive intravascular hemolysis.

Extracellular Hb and hemin are multicomponent triggers of disease processes

Adverse clinical effects that are associated with excessive free Hb can be attributed to a number of specific structural and biochemical properties of the Hb molecule and are caused by the following four mutually interacting mechanisms: (1) extravascular translocation of Hb, which is a principal requirement that Hb and hemin can unleash their adverse reactivity in tissues; (2) nitric oxide and oxidative reactions; (3) release of free hemin; and (4) molecular signaling effects of hemin. These mechanisms are outlined in the subsequent sections and summarized in **Figure 2**.

Mechanism 1: Extravascular translocation of Hb

Following hemolysis, Hb exists in a dynamic equilibrium of tetramer and $\alpha\beta$ -subunit heterodimers, with a predominant dimer state at low plasma Hb concentrations. $\alpha\beta$ -dimers are of a relatively small molecular size (32 kD), allowing for protein translocation and access to vulnerable anatomic sites (e.g. kidneys and the vascular wall). Tissue exposure to Hb is most evident in cases of overt hemoglobinuria following massive intravascular hemolysis, but Hb is also capable to translocate across endothelial barriers, entering subendothelial and peri-vascular spaces and the lymph fluid^{2,3}.

Mechanism 2: Nitric oxide and oxidant reactions

A second mechanism of Hb toxicity is the pro-oxidative reactivity of Hb in plasma or within tissues after extravasation. The Hb reactions with nitric oxide (NO) and with physiologic oxidants (e.g. hydrogen peroxide and lipid peroxides) are the most extensively studied. NO consumption and subsequent Hb oxidation occurs via two reactions: (1) NO dioxygenation of oxy-Hb that generates nitrate (NO_3^-) and ferric Hb (Hb-Fe^{3+}) and (2) iron nitrosylation of deoxy-Hb which occurs by direct iron binding of NO to non-liganded ferrous (Fe^{2+}) Hb.

The current understanding of the biochemistry and pathophysiology of these reactions have

largely been derived from the study of Hb-based oxygen carriers (HBOCs) and their well-documented adverse effects on hemodynamics⁴. Targeted mutagenesis of Hb aimed at limiting interactions with NO or chemical modifications to limit access to sites of NO bioavailability within the vascular wall (e.g. by chemical crosslinking of Hb into large polymers or surface decorated conjugates) attenuate vasoconstriction and hypertension^{5,6}. Therefore, NO depletion by extracellular Hb is now a widely accepted hypothesis to explain the acute hypertensive response that occurs during massive hemolysis (reaching moderate to high plasma levels of free Hb) or during HBOC infusion^{6,7}. Besides vasodilator depletion, another result of Hb-NO reactions might be the generation of ferric Hb within tissue parenchyma. Accumulation of ferric Hb within tissues may promote heme release and/or transfer of heme to other proteins/lipids with secondary toxicity driven by the free heme.

The biochemistry of Hb reaction with peroxides has been scrutinized over the last forty years⁸. Although, the significance of these reactions for Hb and heme driven pathophysiology is still poorly defined. The assumption that oxidative Hb side-reactions could be an important determinant of Hb toxicity was based on observations that peroxides are formed and released into the extracellular space in relatively large quantities during inflammation and ischemia-reperfusion. Under in vitro conditions, peroxide reaction with Hb results in the formation of ferric (Fe^{3+}) Hb, higher oxidation iron species (e.g. ferryl (Fe^{4+}) Hb), and associated radicals (**Figure 2, Mechanism II**). It has been suggested that globin-chain free radicals are available for localized amino acid oxidations (e.g., within Hb) or radical transfer to non-Hb molecules (e.g. lipoproteins)^{9,10}. The proposed final outcome of these reactions is Hb self-destruction, heme loss and globin chain cross-linking/precipitation which can ultimately lead to tissue damage¹¹. It should be noted that the putative impact of these reactions on disease conditions is based on limited and indirect experimental evidence, thus, it remains uncertain whether significant quantities of ferryl (Fe^{4+}) Hb and radicals are generated during in vivo hemolysis and if they contribute to disease. The only oxidized Hb species that can be consistently quantified in vivo are ferric (Fe^{3+}) Hb and hemichrome, a structurally distorted form of ferric (Fe^{3+}) Hb. The disparity between in vitro biochemical observations and in vivo findings may be due to the shifted balance between oxidation and reduction reactions in in vivo¹². The availability of large quantities of small molecule and enzymatic reductants may reduce the stability of higher oxidation state Hb species and Hb-derived radicals to undetectable levels. Intramolecular Hb crosslinks, porphyrin-globin covalent adducts and globin chain amino acid oxidations have been defined as surrogate markers for ferryl (Fe^{4+}) Hb formation. Such modifications were found in Hb recovered from spinal fluid after subarachnoid hemorrhage and from the urine, and suggested that peroxidative reactions may contribute to Hb toxicity in vivo^{13,14}.

Mechanism 3: Hemin release and transfer

A third mechanism of Hb toxicity is through release of hemin from ferric (Fe^{3+}) Hb, which is the main product of the oxidative reactions described in the previous section. Hemin release allows for transfer of the reactive porphyrin to cell membranes or soluble plasma proteins and lipids, and it provides free hemin as a ligand for molecular signaling interactions. As a hydrophobic molecule it is unlikely that significant quantities of free, monomeric hemin can be present in plasma. Therefore, transferred hemin in the form of low-affinity hemin-protein (e.g. hemin-albumin) or hemin-lipid complexes are the most likely physiologic end products of hemin release. Depending on the protein or lipid environment, free iron-protoporphyrin can function as an intermediate and transform the recipient molecule into a reactive end product. The most identifiable toxic end product of hemin release is oxidized low-density lipoprotein (oxLDL)¹⁵. LDL oxidation and associated inflammatory and cytotoxic activities represents a critical example of Hb's ability to induce vascular injury^{16,17}.

Mechanism 4: Molecular signaling effects of hemin

Hemin can selectively bind to a number of receptors, transcription factors and enzymes and thereby alter cell activation state, gene transcription and metabolism. The most well defined interaction is the binding of hemin to the transcriptional repressor Bach-1, which regulates transcription of heme-oxygenase 1 (HO-1) and other anti-oxidant enzymes that are essential for the adaptive response to enhanced intracellular hemin levels¹⁸. Hemin is also a ligand of the nuclear hormone receptor REV-ERB, which regulates circadian rhythm, glucose metabolism, and adipogenesis¹⁹. Inhibition of the proteasome by hemin and by some designed hydrophilic porphyrins is presently documented in biochemical assays only^{20,21}. However, if confirmed in biological systems, this activity may help explain aspects of hemin toxicity. Also, activation of toll-like receptors (TLRs) and downstream inflammatory signaling, particularly of the TLR-4 pathway, can be triggered by free hemin in some models²²⁻²⁴.

In summary, the definitive pathophysiology of extracellular Hb is dependent on timing, quantity and tissue localization of Hb/hemin exposure in a specific clinical condition, and may result from cumulative effects largely described by the four mechanisms discussed above. For example, systemic and to some extent pulmonary hypertension is the most apparent and readily measurable effect of free Hb following intravascular hemolysis. The mechanism(s) driving acute elevations in blood pressure are Hb translocation into subendothelial spaces, local NO depletion within the vessel wall and subsequent vasoconstriction. Vascular complications of chronic and/or intermittent Hb exposure are likely more complicated, involving inflammation, localized oxidative reactions, thrombosis, vascular remodeling and renal impairment²⁵⁻²⁸. As outlined in the following sections, all four mechanisms of Hb and hemin toxicity are specifically attenuated by the natural scavenger proteins haptoglobin (Hp) and hemopexin (Hpx), explaining the extraordinary protective function of

these molecules.

Biochemistry and physiology of Hb and hemin scavenger proteins

During mild to moderate hemolysis, a network of scavenger proteins, receptors, and enzymes accomplishes clearance and detoxification of extracellular Hb and hemin. The primary clearance pathway involves transport to the liver parenchyma or macrophages, breakdown of the porphyrin by the heme-oxygenases into bilirubin, carbon monoxide and iron, and recovery of the iron for de novo erythropoiesis. Scavenger proteins attenuate the toxicity of Hb and hemin, such that these potentially toxic molecules are captured and transported in an inert form. The plasma proteins with promising therapeutic potential are Hp and Hpx. These proteins are discussed in more detail in the following sections.

Haptoglobin

Hp fundamentally changes the biochemical and physiological profile of free Hb²⁹. When bound within the large molecular size Hb:Hp complex (>150 kD), Hb remains sequestered within the intravascular space, translocation of Hb into the kidney and across the endothelial layer appears to be prevented. This simple mechanism keeps potentially adverse biochemical reactions of the free Hb with NO and/or peroxides away from the most susceptible anatomic sites such as the vascular wall. Collectively, intravascular sequestration appears to be the most effective way by which Hp prevents Hb induced hypertension and renal damage (**Figure 3**). Additionally, Hb remains contained within the reducing (i.e. anti-oxidant rich) environment of blood plasma until monocyte and macrophage clearance is complete.

Recent in vitro experiments suggest that Hp may also alter Hb's oxidative reactions. Hp decreases the redox potential of bound ferric (Fe³⁺) Hb, stabilizes the higher oxidation state of Hb ferryl (Fe⁴⁺) Hb and prevents radical transfer to non-Hb molecules in the presence of oxidants^{30,31}. The recently resolved crystal structure of the porcine Hb:Hp complex provided some structural basis for the protection of critical amino acids that are primary targets of globin oxidation³². As a result of this protection, globin oxidation with subsequent protein degradation does not occur when Hb is sequestered in the Hb:Hp complex³³. The structural stability of the complex may prevent accumulation of pro-inflammatory Hb degradation products that can evade clearance by scavenger receptors^{14,34}. Additionally, hemin resides firmly in the Hb:Hp complex, it cannot transfer to hemin acceptors, such as hemopexin, lipoproteins and albumin³⁵. Consequently, prevention of hemin transfer is another essential mechanisms by which Hp protects against Hb-driven oxidation of membrane lipids, plasma lipoproteins, and accumulation of free hemin is prevented.

Genetic heterogeneity of Hp alpha-subunit composition in humans allows for the existence of a structural polymorphism with a dimeric Hp 1-1 ($\alpha_2\beta_2$) and heterogeneous multimeric Hp 2-2 and Hp 2-1 ($\alpha_{>2}\beta_{>2}$) phenotypes³⁶. Each Hp beta chain can bind one Hb $\alpha\beta$ dimer, allowing for two binding sites in Hp 1-1 and multiple Hb dimer binding sites in Hp 2-2. The protein binding capacity

and the basic biochemical functions of Hp 1-1 and Hp 2-2 are equal as they relate to Hb clearance and detoxification (P.W. Buehler and D.J. Schaer, submitted manuscript). The molecular basis for the apparent association of the Hp 2-2 genotype with an enhanced risk for cardiovascular diseases in some high-risk patient populations remains unexplained^{36,37}.

A human plasma-derived Hp product was developed by the Japanese Green Cross (now Benesis Corporation) and was approved in 1985 for the treatment of hemolysis due to extracorporeal circulation, burn injuries, and trauma with massive blood transfusions (**Table 1**). Subsequent findings of several small-scale studies indicate that Hp supplementation could prevent renal tubulus damage during open-heart surgery with cardiopulmonary bypass, the HELLP (Hemolysis Elevated Liver Enzymes Low Platelets) syndrome, and after hemolysis complicating endoscopic injection sclerotherapy of esophageal varices with ethanolamine-oleate (**Table 1**). Case reports and studies in a limited number of patients further support the successful use of Hp in patients with hemolytic anemias (i.e. thalassemia and G6PD deficiency), severe burn injury, in patients with hemolysis resulting from accidental ABO incompatible RBC transfusion, and as a prophylaxis or treatment for hemolysis after ABO mismatched bone marrow transplantation. The primary clinical criteria for Hp administration and dosing in these reports were appearance and reversal of hemoglobinuria, with the intent of limiting renal injury. Reported doses of Hp that were administered for treating acute hemolytic conditions were between $\approx 2\text{g}$ to $>10\text{g}$ per patient, with dosing schedules of repeated 2g (children) or 4g (adults) boluses. The cost for the Hp therapeutic in Japan is currently at 540 US dollars per 2g bolus. To our knowledge there is so far no reported use of Hp in chronic hemolytic conditions where repeated dosing over a longer treatment period might be necessary to effectively suppress free plasma Hb levels.

Hemopexin

Hpx is a hemin-binding plasma glycoprotein, which forms the second line of defense against intravascular hemolysis associated with hemin release from ferric (Fe^{3+}) Hb. Multiple proteins bind hemin, including albumin ($K_d \sim 10^{-8} \text{ M}$) and the lipoprotein particles LDL/HDL ($K_d 10^{-10} \text{ M}$ to 10^{-11} M). However, Hpx ($K_d < 10^{-13} \text{ M}$) is the most effective hemin binding protein that sequesters the porphyrin in an oxidatively inert hexacoordinate conformation in a complex with a 1:1 stoichiometry^{38-39,42}. The most relevant function of Hpx in plasma and tissues is likely the protection of susceptible lipoproteins against oxidative modifications, and to limit hemin interactions with cell surface receptors such as TLR-4^{24,38,43}. In hemin exposure models using the Hpx knockout mouse the scavenger protein demonstrated attenuation of hemin induced endothelial cell activation, vaso-occlusion in the liver, and renal damage^{27,44}. In the same model, data suggested that Hpx shifts plasma hemin to endocytosis and decomposition in hepatocytes.

Like Hp levels, Hpx plasma levels are generally depleted in conditions with intravascular hemolysis, suggesting that supplementation of the plasma protein might provide some benefit to patients⁴⁵. However, no Hpx product is available at a quality that would allow more extensive in

vivo studies beyond murine models. Therefore, expanded studies with Hpx treatment may not be possible in the near future for larger animal models of relevant clinical conditions such as blood transfusion or coronary artery bypass grafting and extracorporeal circulation.

Hb and hemin scavenger receptors

Endocytic receptors clear the biochemically inert circulating Hb:Hp and hemin:Hpx complexes from the circulation. In vitro and ex vivo experiments demonstrated that macrophages and peripheral blood monocytes, respectively, are the primary cell types that clear Hb and Hb:Hp complexes via binding and internalization of the scavenger receptor CD163⁴⁶⁻⁴⁹. The strong induction of CD163 by glucocorticoid treatment in patients with autoimmune diseases identified the Hb scavenger receptor as a potential therapeutic target⁵⁰. Clearance of “endogenous” Hb:Hp is a rapid process following mild hemolysis, however, data obtained in animal models of Hb:Hp infusion or intravascular hemolysis with therapeutic Hp administration suggest slower clearance depending on the level of Hb:Hp accumulation and on the species evaluated. We observed a long circulation half-life of approximately 12 hours for human and canine Hb:Hp complexes in beagle dogs with starting plasma concentrations of $\approx 150 \mu\text{M}$ (heme). The half-life of a human Hb:Hp complex was approximately 16 hours at the same plasma concentration in guinea pigs (unpublished data P.W. Buehler and D.J. Schaer). Recent work indicates that CD163 binding specificity for Hb and the Hb:Hp complex, respectively, has a considerable inter-species variability, and therefore, the threshold for saturation of clearance mechanisms in humans and some laboratory animals might differ significantly⁵¹. Thus, it will be critical for early human trials to define the pharmacokinetics of the Hb:Hp complex at different intravascular concentrations that match conditions of single or repeated Hp administration during acute, intermittent and chronic hemolysis. A similar clearance concept as for the Hb:Hp complex has been proposed for clearance of the hemin-hemopexin complex, which is bound and internalized by the low-density lipoprotein receptor-related protein (LRP)/CD91 on hepatocytes where hemin is degraded by HO-1^{52,53}.

Depletion of Hp and Hpx complexes via their cognate receptors during acute and chronic intravascular hemolysis promotes the pathophysiologic consequences outlined in Figure 1, which we hypothesize could be interrupted by supplementation.

The role of Hb, heme and their scavenger proteins in specific human diseases

Sickle cell disease and other hematological diseases

Hemolysis and vaso-occlusion are the hallmarks of sickle cells disease. Intravascular hemolysis accounts for one third of red cell destruction leading to increases in plasma free Hb and hemin. Back in the 1960s it was recognized that plasma levels of free Hb can be as high as $25 \mu\text{M}$ during sickle cell crisis, with basal plasma Hb levels at $5\text{-}10 \mu\text{M}$ in sickle cell patients. Hp and Hpx levels were found to be depleted⁴⁵. Low Hp and increased plasma free Hb levels were associated with increased protein carbonyl and nitrotyrosine levels in sickle cell anemia⁵⁴. Clinically, uncomplicated

pain episodes were found to be associated with increases in plasma hemoglobin levels^{55,56}, and low levels of Hp correlated with pulmonary hypertension in sickle cell anemia⁵⁷. Although the reported plasma Hb levels in sickle cell patients are much lower than the plasma Hb levels that have been explored in hemodynamic studies of HBOC administration and some animal models of severe hemolysis, these data suggest that free Hb could be a strong pathophysiologic component of the vascular complications of sickle cell disease.

Pathophysiologic hypotheses have focused on the causal relationship between Hb mediated NO consumption, vascular complications and increased pulmonary artery pressure. In these critical studies, an increased tricuspid valve jet regurgitation (TRV) of >2.5m/s was defined as a surrogate marker of pulmonary hypertension in sickle cell patients. Increased TRV was found in 30% of patients with sickle cell disease and was well correlated with markers of hemolysis⁵⁸. Generally, plasma Hb levels in sickle cell patients are orders of magnitude lower than the levels that were studied in classical experimental models of Hb or HBOC mediated NO depletion and vasoconstriction. However, free Hb concentrations of 6 μ M impaired the vasodilatory response to nitroprusside infusion, suggesting that the low free plasma Hb concentrations in sickle cell patients could still critically limit NO bioavailability and promote vasoconstriction, platelet activation and coagulopathy⁵⁹⁻⁶¹. Cumulatively, these effects were hypothesized to be causative for pulmonary hypertension and other vascular complications. More recently, two independent studies from France⁶² and Brazil⁶³ employed right heart catheterization (which is the gold standard for diagnosing pulmonary hypertension) in addition to echocardiography for the evaluation of sickle cell patients. These studies confirmed that a large proportion of sickle patients have increased TRV, but in only 6% and 10% of the patients, respectively, pulmonary hypertension was confirmed by right heart catheterization. Even fewer patients (2.75% and 3.75%) had a pre-capillary pattern of pulmonary hypertension, which would be expected as the typical hemodynamic pattern if NO depletion and vasoconstriction within the pulmonary circulation were the primary pathophysiologic mechanism. In both studies levels of lactate dehydrogenase (LDH) were significantly higher in patients with confirmed pulmonary hypertension. It was therefore concluded that pulmonary hypertension in sickle cell patients is caused by a complex, multi-factorial pathophysiology which may involve NO depletion, vasoconstriction, thromboembolic vascular occlusion, left ventricular impairment, chronic vascular remodeling, as well as secondary hemodynamic effects caused by the hyperdynamic circulation that occurs in many patients with more severe or chronic anemia^{64,65}. The quantitative contributions of these individual factors and the specific role of free Hb and hemin in the pathophysiology of vascular complication in sickle cell disease remain an important area for further mechanistic evaluation in clinical settings.

Murine models of sickle cell disease continue to provide support for a critical role of hemolysis and free Hb and hemin, respectively, in vaso-occlusive complications⁶⁶. Sickle mice have intravascular hemolysis as evidenced by elevated plasma Hb, decreased Hp and Hpx, elevated reticulocytes and plasma bilirubin. In studies using a model of vascular stasis, induction or

over-expression of heme oxygenase-1 inhibits vascular stasis induced by hypoxia⁶⁷⁻⁶⁹. Furthermore, infusion of hemoglobin or heme into sickle mice induced vascular stasis that could be inhibited by Hp or Hpx supplementation⁷⁰. In the pro-oxidative environment of sickle cell anemia, ferrous Hb may react with NO or other oxidants to generate ferric Hb from which heme is released⁶⁹. In the absence of Hp and Hpx, plasma heme can interact with albumin or other plasma proteins and/or lipoproteins, but ultimately heme is distributed to cells in the vasculature including endothelial cells and leukocytes. Heme's hydrophobicity allows it to enter cell membranes and activate TLR4 signaling pathways, which trigger pro-inflammatory and pro-thrombotic responses thereby promoting blood cell adhesion and vaso-occlusion^{24,70}.

Collectively, epidemiologic and experimental data support a concept whereby substitution of Hp and/or Hpx could attenuate or prevent complications associated with accelerated intravascular hemolysis in sickle cell disease.

There are many other hematologic diseases in which hemolysis constitutes a primary disease process (e.g. thalassemia, paroxysmal nocturnal hemoglobinuria (PNH), glucose-6-phosphate dehydrogenase deficiency) or a secondary phenomenon (e.g. hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP)). Although the contribution of free Hb and heme toxicity to disease outcome has not been explored in this wider arena of hemolytic disorders, the broader availability of Hp and Hpx will provide us the required tools to systematically explore the therapeutic potential of Hb/heme neutralization in these multi-factorial diseases.

Blood transfusion

Retrospective data analyses suggest that transfusion of older stored blood (approaching 42 days) results in increased morbidity and mortality when compared to newer blood⁷¹. Increased morbidity has typically been associated with immune suppression, infection, cardiovascular complications and acute/chronic kidney injury. One of the most essential consequences of the so-called "RBC storage lesion" is related to reduced deformability, enhanced in vivo fragility and susceptibility of RBCs to attack by components of the immune system. These changes may be accompanied by increased hemolysis of older storage RBC.

Although the clinical impact of blood storage duration on clinical outcome remains controversial, numerous models have been developed to evaluate the effects of hemolysis and Hb toxicity⁷²⁻⁷⁵. Guinea pigs subjected to massive transfusion (the equivalent of >10 units) of maximally stored guinea pig blood demonstrated increased plasma Hb concentrations over a 24-hour period, which resulted from post-transfusion hemolysis⁷⁶. This was associated with increased hemoglobinuria, acute renal failure, hypertension and vascular injury. Hp co-infusion with older storage blood resulted in attenuation of adverse renal and vascular effects to resemble that of new blood transfusion. These observations are supported by several studies that suggest blood transfusion in mice, rats and sheep are associated with increased blood pressure, NO depletion,

and increased plasma Hb concentrations^{74,75,77}. Hb related transfusion complications in humans have also been associated with ABO mismatch and massive transfusion (>10 units/24 hours).

Clinically, Hp has been used to treat adverse transfusion outcomes in Japan, and the primary reported outcome of therapeutic Hp treatment appears to be prevention of renal tubular injury (**Table 1**).

Sepsis and Malaria

Hemolysis can also occur during severe bacterial infection and sepsis, and depletion of Hpx has been associated with more severe disease and fatal outcome⁷⁸. Within these complex disease processes, hemin could act as an activator of innate immunity via pattern recognition receptor activation (e.g. TLR-4), via synergistic effects with microbial products or endogenous pro-inflammatory ligands (e.g. HMGB1), or via peroxidative tissue damage^{24,79,80}. Studies on mice suggested that the net effect of free hemin in polymicrobial sepsis could be detrimental and that neutralization of hemin by Hpx administration ameliorates this effect⁷⁸.

The protective action of Hpx in sepsis may involve the control of excessive hemin peroxidative activity, inhibition of innate immunity receptor interactions and redirection of free hemin away from susceptible tissues towards clearance in the liver. Restriction of hemin-iron access by Hb and hemin scavenger proteins could be another protective mechanism against severe infections by some bacterial strains^{81,82}. The in vivo activity of Hpx may also involve Hpx activities that are independent of its primary hemin scavenger function. The outcome of such activities has been noted as anti-inflammatory in some instances (e.g. Hpx attenuates inflammatory cytokine secretion by LPS stimulated macrophages⁸³), whereas impaired bacterial clearance was found as a consequence of suppressed neutrophil migration in other mouse studies⁸⁴. At this point, it is unknown whether such non-hemin related effects of Hpx are an inherent activity of the protein itself or side effects caused by impurities or degradation products in some preparations of plasma-derived Hpx⁸⁵.

In mouse models of malaria, Hb/hemin driven oxidative damage and NO depletion were linked to the evolution of cerebral malaria⁸⁶. However, although we may suspect that this pathophysiology could be attenuated by Hp and/or Hpx treatment, the efficacy of scavenger proteins has not yet been tested for this specific condition.

Conclusion: Is there an unmet need for scavenger protein-based therapeutics?

Multiple hematologic and non-hematologic disease states are associated with RBC lysis and the adverse effects of free Hb and hemin potentially complicate clinical disease outcome. There is currently no viable therapy designed to attenuate the adverse effects of free Hb and hemin. Intuitively, replacement or supraphysiologic dosing of Hp and/or Hpx to match acute or ongoing hemolysis may be of therapeutic benefit by attenuating many of the pathophysiologies that we discuss in this perspective, and a number of clinical situations where Hb contributes to acute (e.g.

acute kidney injury) and chronic (e.g. vascular remodeling) sequelae. Plasma purified Hp has been marketed in Japan since 1985 with primary indications for use in conjunction with extracorporeal circulation, massive transfusion and thermal injury. The primary therapeutic effect in these disease states is protection of kidneys from Hb induced toxicity⁸⁷. Thus far no therapeutic experience exists for Hpx in a clinical setting. However, several US and EU based pharmaceutical companies have commenced development projects to fractionate Hp and Hpx from human plasma for use as therapeutics during hemolytic diseases. In 2011 a human plasma-derived Hp was given orphan drug status for the treatment of sickle cell disease in the EU. However, to date preclinical proof of concept for Hp and Hpx have been studied within a general context of hemolysis and Hb driven pathophysiology and not with intent to treat a specific disease. Within most countries drugs/biologics are approved based on indication specific improvement in disease outcome and acceptable safety. Therefore, rational selection of potential indications, dose schedules and clinical trial design to achieve measurable outcomes and acceptable safety profiles will be critical for the concepts presented here to successfully progress towards improved patient care. These and other questions will pose exciting basic science, clinical and regulatory challenges in development process of Hb/hemin scavenging therapeutics.

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Table 1. Literature cited clinical use of Haptoglobin in Japan

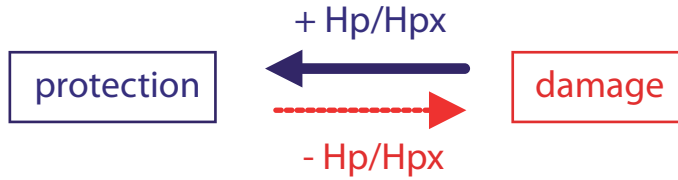
Clinical Condition	Subjects/Controls	Haptoglobin dose	Primary finding (intention of treatment)	Reference
Cardiopulmonary bypass surgery	16/21	6 grams	Prevention of acute renal failure	87
	14/0	4 grams		88
CABG in a patient with β -thalassemia and severe hemolysis	Single case report			89
Peripheral blood stem cell transplant	Single case report	3 grams	Prevention of acute renal failure	90
Hemolytic transfusion reaction	Single case report	6 grams	Prevention of acute renal failure	91
Massive transfusion	16/34	6 grams	Undetermined benefit	92
Extracorporeal circulation	10/10	6 grams	Prevention of acute renal failure	93
HELLP syndrome (Hemolysis Elevated Liver enzymes Low Platelets)	17/17	3 grams	Prevention of acute renal failure	94
ABO incompatible bone marrow transplantation	Two patient case reports	11 grams	Prevention of acute renal failure	95
Thermal injury	Single case report (repeated, large dose therapy)	>20 grams	Improved outcome	96
			Prevention of hemoglobinuria and acute renal failure	97
Glucose-6-phosphate dehydrogenase deficiency	Single patient case report (24 month old boy)	8.5 grams	Prevention of hemoglobinuria	98
Adult patent ductus arteriosus, coil embolization	Single patient case report	45 grams	Prevention of hemoglobinuria	99

Figure Legends

Figure 1: Schematic summary of the Hb clearance compartments and the main acute and chronic pathologies that can be associated with intravascular hemolysis. The availability of the Hb and heme scavenger proteins haptoglobin (Hp) and hemopexin (Hpx) shifts the physiologic balance from tissue damage towards protection.

Figure 2: Schematic summary of the principal mechanisms of hemoglobin toxicity and protection by the plasma scavenger proteins haptoglobin and hemopexin.

Figure 3. Haptoglobin sequestration of hemoglobin: Guinea pigs were infused with stroma free Hb (peak plasma Hb concentration 150 μ M heme) and after 10 min a treatment group of animals was infused with human plasma derived haptoglobin (Hp) to match an equimolar Hb:Hp concentration. (A) Mean arterial blood pressure response before and after Hp treatment. (B) Unbound plasma Hb (red) prior to Hp administration and after Hp administration. The left shift in the chromatogram indicates the large molecular size Hb:Hp complex with approximately 90% Hp bound and 10% unbound Hb. (C) Hemoglobinuria following Hb infusion (150 μ M heme) -Hp (left) and +Hp (right). (D) Iron deposition (brown staining) in normal kidney renal cortex (left), Hb infusion (middle) and Hb infusion + Hp (right). (E) Heme oxygenase-1 expression in kidneys after 24 hours following Hb exposure with and without Hp infusion (left), densitometry is shown to the right of the HO-1 western blot. All data are presented as mean \pm SEM.



Vascular

Acute Effects of Hemolysis

NO depletion + Oxidative stress
Hypertension
Vaso-occlusion

Chronic Effects of Hemolysis

Endothelial dysfunction
Vascular injury
Lipoprotein oxidation
Atherosclerosis

Hepatic

Chronic Effects of Hemolysis

Dysregulated iron metabolism

Renal

Acute Effects of Hemolysis

Renal Hb exposure
Oxidative damage

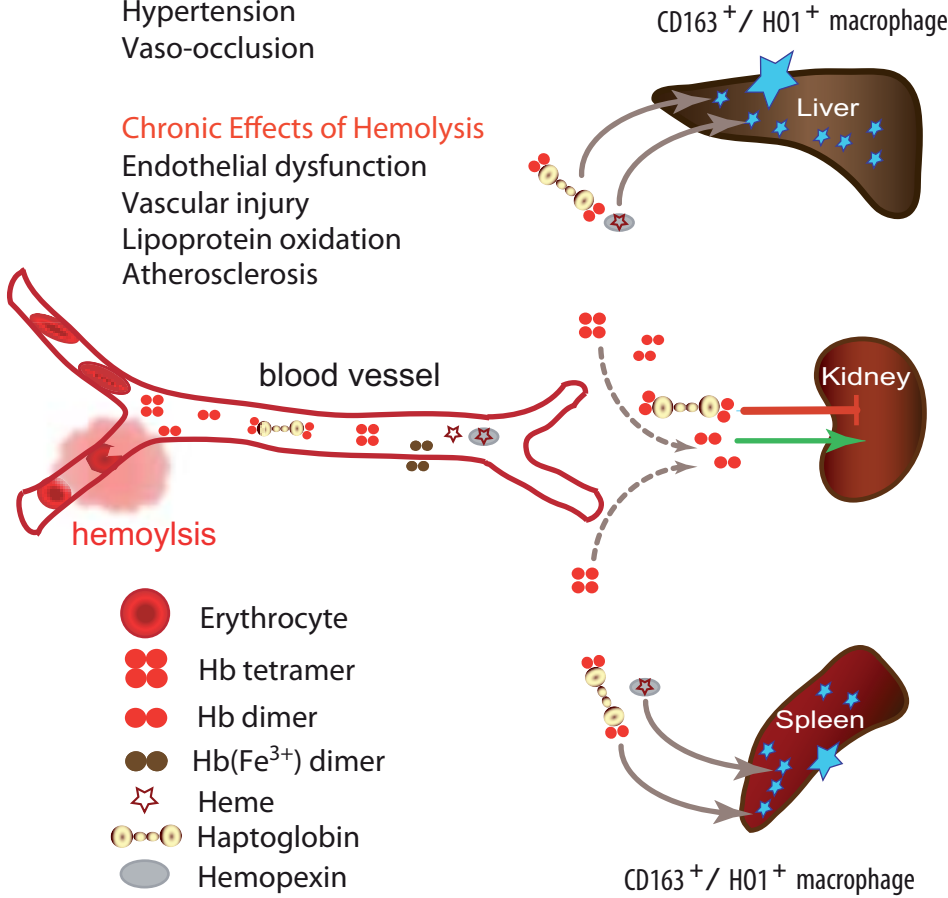
Chronic Effects of Hemolysis

Tubular injury
Renal failure

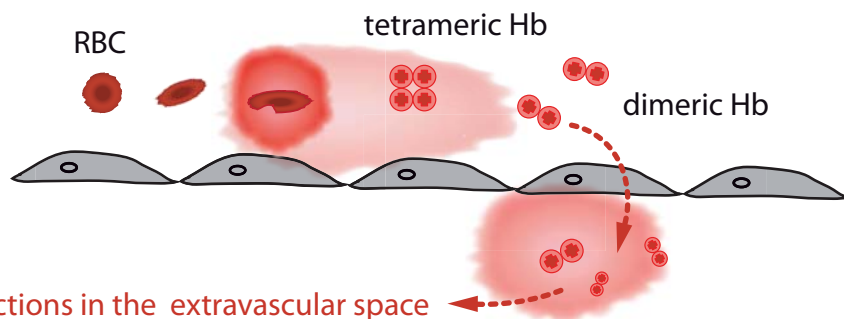
Splenic

Chronic Effects of Hemolysis

Extramedullary hematopoiesis
Immune Dysregulation

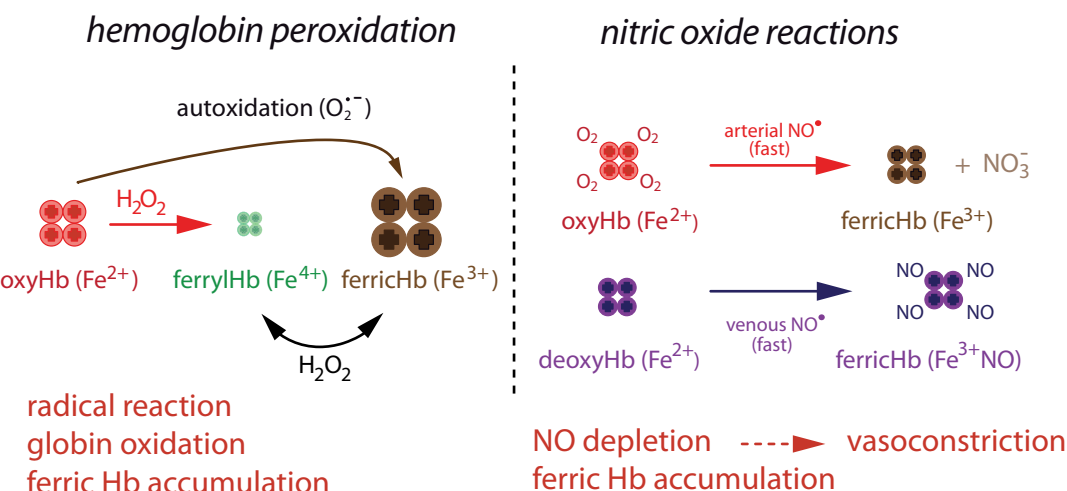


Mechanism I: Extravascular translocation of Hb



Haptoglobin:
Hb sequestration
in the intravascular
space

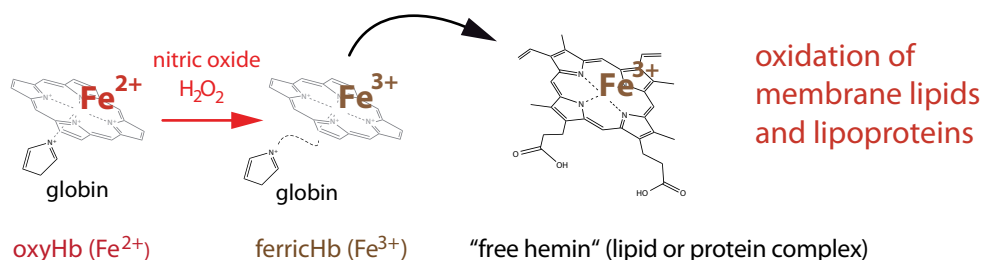
Mechanism II. Nitric oxide and oxidant reactions



Haptoglobin:
control of
radical reactions
and
radical translocation

Haptoglobin:
limits NO depletion
in the vascular wall

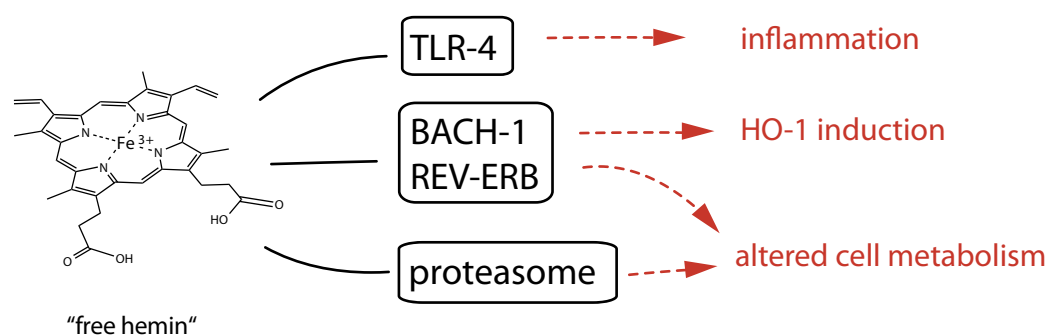
Mechanism III. Hemin release and transfer



Haptoglobin:
prevents
hemin release

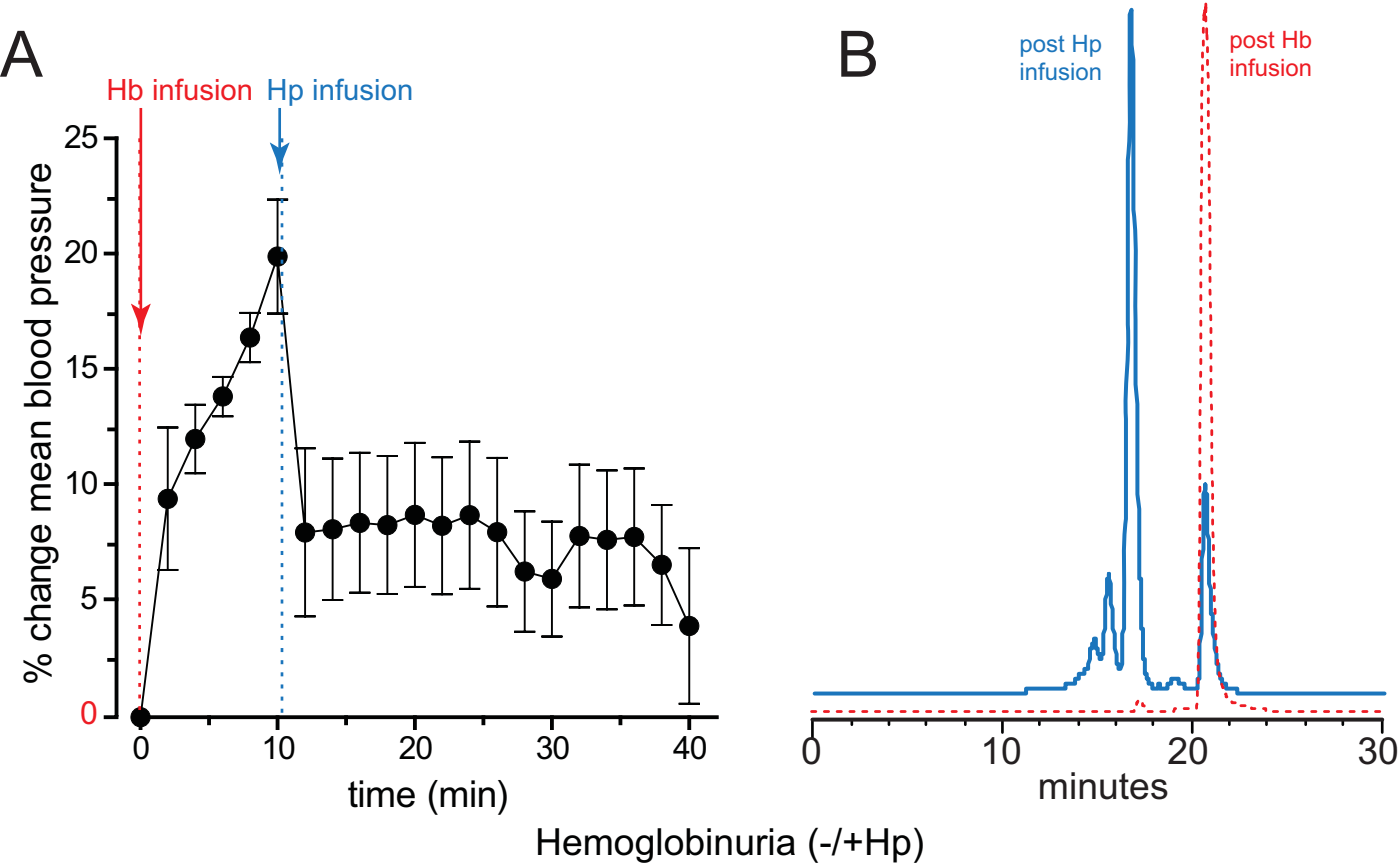
Hemopexin:
oxidative neutralization
of free hemin

Mechanism IV. Molecular signalling effects of hemin



Hemopexin:
blocks interaction
with cell surface receptors

Hemopexin:
prevents intracellular
translocation



Hemoglobinuria (-/+Hp)

